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Regular Article

Synthesis and biological evaluation of MS-347a derivatives against plant pathogenic fungi based on a strategy of *laeA* gene introduction

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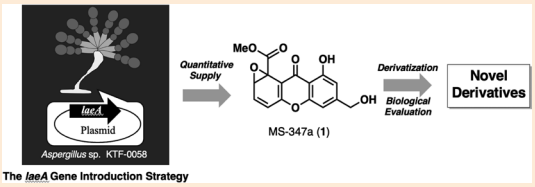
Supplementary material

Our group previously identified MS-347a (**1**) as a new fungicide candidate from the culture broth of the mutant strain, *Aspergillus* sp. KTF-0058, which had the *laeA* gene inserted. This mutant strain was able to produce a sufficient supply of **1**, allowing for its use to investigate the structure–activity relationship. To this end, we synthesized 11 derivatives and evaluated their antifungal activity. Among these derivatives, the aldehyde derivative exhibited superior antifungal potency as compared to **1**, and some acyl derivatives were able to maintain the antifungal activity of **1** despite significant structural changes. From these results, it is found that the aldehyde derivative is one of the most promising fungicidal candidates, and the introduction of acyl groups could be utilized to create chemical probes for target-identification experiments.



Keywords: MS-347a, Derivatization, Natural products, Fungicide candidates, LaeA, *Aspergillus* sp.

Introduction

As the world's population continues to grow, global food security becomes ever more important. Whether through climate change, conflicts, or infectious disease, the global food supply is under great strain and is vulnerable to collapse. As such, our group has been aiming to tackle fungal plant pathogens with fungicidal natural products to ensure and maintain the food supply. In fact, pathogenic plant fungi have already caused significant damage to humanity's essential food crops, and that damage has been increasing.[1,2)] The rapid emergence of drug-resistant pathogenic plant fungi to several conventional fungicides is a major contributing factor to the increasing damage to crops, and this resistance could be attributed to the highly plastic genomes of fungi.[3)] Considering this, it is likely preferable to use multiple fungicides with unique mechanisms of action against these pathogens to minimize the emergence of drug resistances. Therefore, continuous screening for new fungicide

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candidates is in high demand for efficient control of pathogenic plant fungal disease. Accordingly, we conducted screening of microbial secondary metabolites and found that the culture broth of *Aspergillus* sp. FKI-5362 strain demonstrated antifungal activity. However, we were not able to initially isolate and identify the active compound due to its extremely low production. To settle this challenge, we introduced the putative methyltransferase *laeA*, known as a global regulator for expressions of secondary metabolite biosynthetic genes, into the strain.[4,5] The *laeA* gene introduced mutant (KTF-0058) strain produced the target compound in ten-folds greater quantity compared to the wild strain, enabling the identification of MS-347a (**1**) as a fungicidal compound (Fig. 1). Ultimately, we revealed that **1** showed potent antifungal activity against broad plant pathogenic fungal species. In this study, we synthesized new derivatives from **1** which was produced under the *laeA* gene introduction strategy to elucidate the structure–activity relationship of **1** for antifungal activity against pathogenic plant fungi, as well as to develop more potent fungicide candidates.

Materials and methods

1. General experimental procedures

High- and low-resolution mass spectra were obtained using an AB Sciex QSTAR Hybrid LC/MS/MS Systems (AB Sciex, Framingham, MA, USA) and JEOL JMS-T100LP (JEOL, Tokyo, Japan). NMR spectra were measured using AVANCE III HD-600 (Bruker, CA, USA), and Varian-INOVA600 with ^{1}H NMR and ^{13}C NMR at 600 MHz and 150 MHz, respectively, and JEOL JNM-ECA-500 (JEOL, Tokyo, Japan), with ^{1}H NMR and ^{13}C NMR at 500 MHz and 125 MHz in DMSO-d_6 and $CDCl_3$. The chemical shifts are reported in ppm and referenced to DMSO-d_6 (2.50 ppm) and $CDCl_3$ (7.26 ppm) in the ^{1}H NMR spectra and DMSO-d_6 (39.50 ppm) and $CDCl_3$ (77.16 ppm) in the ^{13}C NMR.

2. Fermentations of Aspergillus *sp. KTF-0058 strain*

One loop of *Aspergillus* sp. FKI-5362 and KTF-0058 strains grown on an LcA slant (0.1% glycerol, 0.08% KH_2PO_4, 0.02% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% KCl, 0.2% $NaNO_3$, 0.02% yeast extract, and 1.5% agar, pH 6.0) was inoculated into 10 mL of a seed culture medium (2% glucose, 0.2% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% polypepton (Nihon Pharmaceutical Co., Tokyo, Japan), 0.1% KH_2PO_4, and 0.1% agar, pH 6.0) and incubated on a rotary shaker (210 rpm) at 27°C for 3 days. One liter of the seed culture was inoculated into a production medium (10 kg of water-sodden rice). Static fermentation was continued at 27°C for 14 days.

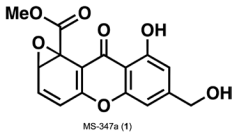


Fig. 1. Structure of MS-347a (**1**)

3. Isolations of MS-347a (*1*)

MS-347a (**1**): The culture of KTF-0058 strain was extracted with 10 L of methanol. After filtration, the filtrate was concentrated *in vacuo* to remove the methanol. The concentrated extract (2 L) was applied on an ODS column (500 mL of resin, YMC Co. Ltd., Kyoto, Japan), which was eluted stepwise with 0, 20, 40, 60, 80, and 100% of MeOH aqueous solutions (each 1 L). The 40% MeOH fraction was concentrated to dryness to afford a crude extract. The extract was chromatographed on a silica gel column and eluted stepwise with a mixture of $CHCl_3$/MeOH (100/0, 100/1, 100/3, 100/5, 100/10, 100/100, and 0/100). After concentrating 100/0 and 100/1 fractions *in vacuo*, the compound which was insoluble in MeOH was washed 5 times with 100 μL of methanol by centrifugation at 10,000 $\times g$ for 1 min, isolating compound **1**. HR-ESIMS *m*/*z* 317.0662 $[M+H]^+$ (calcd *m*/*z* 317.0661 $[M+H]^+$, molecular formula $C_{16}H_{12}O_7$).

4. Synthesis of MS-347a derivatives

General procedure for derivatives **2**, **6**, **7** and **12**

To a stirred solution of **1** in pyridine was added acetic anhydride, propionic anhydride, butyric anhydride, or BzCl at rt. After 24 hr, the reaction solution was evaporated by nitrogen blowdown. The residual oil was purified by PTLC (hexane : acetone = 3 : 2 or *n*-hexane : AcOEt = 1 : 2) to afford acylated MS-347a.

5. Acetyl derivative 2

A reaction of **1** (20.0 mg, 63.2 μmol) with acetic anhydride (149 μL, 1.54 mmol) in pyridine (200 μL, 2.43 mmol), followed by PTLC purification gave acetyl derivative **2** (6.6 mg, 26%) as a yellow solid. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 7.57 (d, J = 1.8 Hz, 1H), 7.20 (d, J = 1.8 Hz, 1H), 7.17 (dd, J = 12.3, 4.9 Hz, 1H), 6.91(dd, J = 12.3, 4.9 Hz, 1H), 5.22 (s, 2H), 4.28 (dd, J = 4.9, 2.0 Hz, 1H), 3.66 (s, 3H), 2.31 (s, 3H), 2.13 (s, 3H); and ^{13}C-NMR (125 MHz, DMSO-d_6) δ 173.5, 170.3, 169.1, 166.8, 158.1, 155.7, 148.7, 144.4, 137.7, 125.6, 118.5, 115.2, 114.8, 114.6, 63.9, 56.9, 56.1, 52.5, 20.9, 20.7. HRMS (ESI+) *m*/*z* calcd for $C_{20}H_{17}O_9$ ($M+H^+$): 401.0873, found: 401.0872.

6. Aromatized derivative 3

To a stirred solution of **1** (20.0 mg, 63.2 μmol) in MeOH (632 μL, 0.1 M) was added Pd/C (2.0 mg, 10% wt of SM) at room temperature. The reaction mixture was evacuated and backfilled with H_2, which was reaerated a total ten times. After being stirred for 1 hr, the reaction mixture filtered through a pad of celite, and the filtrate was concentrated under reduced pressure. The residue was purified by PTLC (hexane : AcOEt = 6 : 1) to give aromatized derivative **3** (8.2 mg, 43%) as a yellow solid. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 12.05 (s, 1H), 7.94 (dd, J = 10.8, 9.0 Hz, 1H), 7.76 (dd, J = 10.3, 1.3 Hz, 1H), 7.45 (dd, J = 10.3, 1.3 Hz, 1H), 7.03 (d, J = 1.6 Hz, 1H), 6.78 (dd, J = 1.6 Hz, 1H), 5.59 (t, J = 7.3 Hz, 1H), 4.60 (d, J = 6.9 Hz, 2H), 3.89 (s, 3H); and ^{13}C-NMR (125 MHz, DMSO-d_6) δ 180.3, 168.8, 160.6, 155.7, 155.5, 154.6, 136.1, 133.0, 123.0, 119.8, 116.7, 107.8, 107.1, 104.3, 62.4, 52.8. HRMS

(ESI+) *m/z* calcd for $C_{16}H_{13}O_6$ ($M+H^+$): 301.0712, found: 301.0718.

7. Methyl derivative 4

To a stirred solution of **1** (20.0 mg, 63.2 μmol) in DMF (632 μL, 0.1 M) were added K_2CO_3 (52.4 mg, 379.2 μmol) and MeI (19.6 μL, 316.0 μmol) at room temperature. After being stirred for 12 hr, the reaction mixture was quenched with sat. NH_4Cl aq. and diluted with AcOEt. The resulting mixture was extracted with AcOEt and washed with brine. The organic phase was dried over Na_2SO_4, concentrated under the reduced pressure. The residue was purified by PTLC (AcOEt) to give methyl derivative **4** (9.1 mg, 44%) as a yellow solid. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 7.08 (dd, J=4.8, 11.9 Hz, 1H), 7.07 (d, J=1.8 Hz, 1H), 6.97 (d, J=1.8 Hz, 1H), 6.85 (dd, J=11.9, 2.1 Hz, 1H), 5.58 (t, J=7.3 Hz, 1H), 4.61 (d, J=7.3 Hz, 2H) 4.23 (dd, J=4.8, 1.8 Hz, 1H), 3.86 (s, 3H), 3.69 (s, 3H); and ^{13}C-NMR (125 MHz, DMSO-d_6) δ 173.8, 167.1, 159.1, 156.8, 156.6, 151.2, 136.4, 125.8, 115.0, 111.6, 106.6, 105.3, 62.3, 57.1, 56.3, 53.2, 52.5. HRMS (ESI+) *m/z* calcd for $C_{17}H_{15}O_7$ ($M+H^+$): 3310818, found: 331.0818.

8. Aldehyde derivative 5

To a stirred solution of **1** (20.0 mg, 63.2 μmol) in DCM (632 μL, 0.1 M) was added MnO_2 (54.9 mg, 632 μmol) at room temperature. After being stirred for 3 hr, the reaction mixture filtered through a pad of celite, and the filtrate was concentrated under reduced pressure. The residue was purified by PTLC (hexane : AcOEt = 1 : 2) to give aldehyde derivative **5** (7.0 mg, 34%) as a yellow solid. ^{1}H-NMR (600 MHz, $CDCl_3$) δ 12.33 (s, 1H) 10.02 (s, 1H), 7.42 (d, J=1.3 Hz, 1H), 7.31 (d, J=1.3 Hz, 1H), 7.08 (dd, J=9.9, 4.2 Hz, 1H), 6.70 (dd, J=9.9, 1.3 Hz, 1H), 4.14 (dd, J=4.2, 1.9 Hz, 1H), 3.90 (s, 3H); and ^{13}C-NMR (150 MHz, DMSO-d_6) δ 192.7, 180.8, 166.9, 161.2, 160.7, 155.8, 141.9, 140.0, 126.3, 113.4, 111.5, 109.4, 57.1, 56.5(2C), 29.5. HRMS (ESI+) *m/z* calcd for $C_{16}H_{11}O_7$ ($M+H^+$): 315.0505, found: 315.0509.

9. Propionyl derivative 6

A reaction of **1** (19.7 mg, 62.2 μmol) with propionic anhydride (200 μL, 1.54 mmol) in pyridine (200 μL, 2.43 mmol), followed by PTLC purification gave propionyl derivative **6** (5.8 mg, 22%) as a white solid. ^{1}H-NMR (600 MHz, $CDCl_3$) δ 7.34 (d, J=1.8 Hz, 1H), 7.01 (d, J=1.8 Hz, 1H), 6.94 (dd, J=9.9, 4.2 Hz, 1H), 6.71 (dd, J=9.9, 1.8 Hz, 1H), 5.19 (s, 2H), 4.07 (dd, J=3.6, 1.8 Hz, 1H), 3.82 (s, 3H), 2.76 (dd, J=17.1, 7.2 Hz, 1H), 3.74 (dd, J=17.1, 7.2 Hz, 1H), 2.44 (q, J=7.2 Hz, 2H), 1.28 (t, J=7.2 Hz, 3H), 1.19 (t, J=7.2 Hz, 3H); and ^{13}C-NMR (150 MHz, $CDCl_3$) δ 174.0, 173.8, 173.0, 167.4, 157.7, 156.4, 149.8, 143.3, 135.7, 126.2, 118.7, 116.3, 115.8, 114.6, 64.4, 57.4, 56.6, 53.0, 27.7, 27.6, 9.18, 8.88. HRMS (ESI+) *m/z* calcd for $C_{22}H_{20}O_9Na$ ($M+Na^+$): 451.1011, found: 451.1005.

10. Butyryl derivative 7

A reaction of **1** (20.0 mg, 63.2 μmol) with butyric anhydride (200 μL, 1.20 mmol) in pyridine (200 μL, 2.43 mmol), followed by PTLC purification gave butyryl derivative **7** (6.3 mg, 22%) as a white solid. ^{1}H-NMR (600 MHz, $CDCl_3$) δ 7.34 (d, J=1.2 Hz, 1H), 6.99 (d, J=1.8 Hz, 1H), 6.94 (dd, J=9.6, 3.6 Hz, 1H), 6.70 (dd, J=9.6, 1.2 Hz, 1H), 5.18 (s, 2H), 4.07 (dd, J=3.6, 1.8 Hz, 1H), 3.81 (s, 3H), 2.72 (dd, J=16.5, 7.5 Hz, 1H), 2.67 (dd, J=16.5, 7.5 Hz, 1H), 2.40 (t, J=7.2 Hz, 2H), 1.84–1.78 (m, 2H), 1.73–1.67 (m, 2H), 1.06 (t, J=7.2 Hz, 3H), 0.97 (t, J=7.2 Hz, 3H); and ^{13}C-NMR (150 MHz, $CDCl_3$) δ 173.8, 173.2, 172.2, 167.4, 157.7, 156.4, 149.7, 143.3, 135.7, 126.3, 118.8, 116.3, 115.8, 114.6, 64.3, 57.5, 56.6, 53.0, 36.1 (2C), 18.5, 18.1, 13.9, 13.8. HRMS (ESI+) *m/z* calcd for $C_{24}H_{24}O_9Na$ ($M+Na^+$): 479.1324, found: 479.1319.

11. Cyclopropanecarbonyl derivative 8

To a stirred solution of **1** (20.0 mg, 63.2 μmol) in DCM (632 μL, 0.1 M) were added Et_3N (29.1 μL, 208.6 μmol) and cyclopropanecarbonyl chloride (18.9 μL, 208.6 μmol) at room temperature. After being stirred for 12 hr, the reaction mixture was quenched with sat. NH_4Cl aq. and diluted with AcOEt. The resulting mixture was extracted with AcOEt and washed with brine. The organic phase was dried over Na_2SO_4, concentrated under the reduced pressure. The residue was purified by PTLC (hexane : AcOEt = 1 : 2) to give cyclopropanecarbonyl derivative **8** (28.0 mg, 95%) as a yellow solid. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.34 (d, J=1.7 Hz, 1H), 7.03 (d, J=1.7 Hz, 1H), 6.93 (dd, J=9.9, 3.8 Hz, 1H), 6.69 (dd, J=9.9, 1.7 Hz, 1H), 5.18 (s, 2H), 4.07 (dd, J=5.4, 1.6 Hz, 1H), 3.83 (s, 3H), 1.99–1.94 (m, 1H), 1.74–1.59 (m, 1H), 1.30–1.2.0 (m, 2H), 1.09–1.05 (m, 4H), 0.96–0.91 (m, 2H); and ^{13}C-NMR (150 MHz, $CDCl_3$) δ 174.6, 173.8, 173.2, 167.4, 157.7, 156.3, 149.7, 143.3, 135.7, 126.3, 118.8, 116.5, 115.8, 114.6, 64.5, 57.5, 56.6, 53.0, 13.4, 12.9, 9.3(2C), 9.1(2C). HRMS (ESI+) *m/z* calcd for $C_{24}H_{21}O_9$ ($M+H^+$): 453.1186, found: 453.1188.

12. Cyclobutanecarbonyl derivative 9

To a stirred solution of **1** (20.0 mg, 63.2 μmol) in DCM (632 μL, 0.1 M) were added Et_3N (29.1 μL, 208.6 μmol) and cyclobutanecarbonyl chloride (23.8 μL, 208.6 μmol) at room temperature. After being stirred for 12 hr, the reaction mixture was quenched with sat. NH_4Cl aq. and diluted with AcOEt. The resulting mixture was extracted with AcOEt and washed with brine. The organic phase was dried over Na_2SO_4, concentrated under the reduced pressure. The residue was purified by PTLC (hexane : AcOEt = 1 : 2) to give cyclobutanecarbonyl derivative **9** (5.2 mg, 17%) as a yellow solid. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.32 (d, J=1.6 Hz, 1H), 6.98 (d, J=1.6 Hz, 1H), 6.94 (dd, J=9.9, 3.9 Hz, 1H), 6.70 (dd, J=9.9, 1.6 Hz, 1H), 5.17 (s, 2H), 4.07 (dd, J=3.9, 1.6 Hz, 1H), 3.81 (s, 3H), 3.56–3.49 (m, 1H), 3.27–3.21 (m, 1H), 2.65–2.54 (m, 2H), 2.38–2.21 (m, 6H), 2.10–1.89 (m, 4H); and ^{13}C-NMR (125 MHz, $CDCl_3$) δ 175.0, 173.8, 173.7, 167.4, 157.7, 156.4, 143.4, 135.7 (2C), 126.3 (2C), 118.7, 114.6 (2C), 64.4, 57.5, 56.6, 53.0, 38.3, 38.0, 29.8, 25.4 (2C), 25.2, 18.6 (2C). HRMS (ESI+) *m/z* calcd for $C_{26}H_{25}O_9$ ($M+H^+$): 481.1499, found: 481.1488.

13. Cyclopentanecarbonyl derivative 10

To a stirred solution of **1** (20.0 mg, 63.2 μmol) in DCM (632 μL, 0.1 M) were added Et_3N (29.1 μL, 208.6 μmol) and cyclobutanecarbonyl chloride (25.4 μL, 208.6 μmol) at room temperature. After being stirred for 12 hr, the reaction mixture was quenched with sat. NH_4Cl aq. and diluted with AcOEt. The resulting mixture was extracted with AcOEt and washed with brine. The organic phase was dried over Na_2SO_4, concentrated under the reduced pressure. The residue was purified by PTLC (hexane : AcOEt = 1 : 2) to give cyclobutanecarbonyl derivative **10** (12.0 mg, 37%) as a yellow solid. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.32 (s, 1H), 7.00 (s, 1H), 6.94 (dd, J=9.9, 1.3 Hz, 1H), 6.71 (dd, J=9.9, 1.3 Hz, 1H), 5.16 (s, 2H), 4.07 (dd, J=3.6, 1.8 Hz, 1H), 3.80 (s, 3H), 3.14 (quin, J=7.9 Hz, 1H), 2.84 (quin, J=7.9 Hz, 1H), 2.14–2.04 (m, 4H), 1.98–1.91 (m, 3H), 1.85–1.58 (m, 9H); and ^{13}C-NMR (125 MHz, $CDCl_3$) δ 176.4, 175.1, 173.8, 167.4, 157.7, 156.4, 149.9, 143.5, 135.7, 126.3, 118.7, 116.5, 115.8, 114.5, 64.4, 57.5, 56.6, 53.0, 44.0, 43.8, 30.2, 30.1, 29.8 (2C), 26.0 (2C), 25.9 (2C). HRMS (ESI-) m/z calcd for $C_{28}H_{27}O_9$ $(M-H)^-$: 507.1655, found: 507.1633.

14. Benzoyl derivative 11

A reaction of **1** (20.0 mg, 63.2 μmol) with BzCl (32 μL, 270 μmol) in pyridine (200 μL, 2.43 mmol), followed by PTLC purification gave benzoyl derivative **12** (5.1 mg, 15%) as a white solid. ^{1}H-NMR (600 MHz, $CDCl_3$) δ 8.22 (dd, J=7.9, 1.2 Hz, 2H), 8.12–8.11 (m, 2H), 7.65–7.63 (m, 1H), 7.62–7.59 (m, 1H), 7.51 (t, J=7.9 Hz, 2H), 7.50–7.47 (m, 3H), 7.24 (d, J=1.2 Hz, 1H), 6.93 (dd, J=9.6, 3.6 Hz, 1H), 6.72 (dd, J=9.6, 1.2 Hz, 1H), 5.48 (s, 2H), 4.05 (dd, J=3.6, 1.2 Hz, 1H), 3.65 (s, 3H); and ^{13}C-NMR (150 MHz, $CDCl_3$) δ 173.7, 167.3, 166.2, 165.3, 157.7, 156.4, 149.9, 143.3, 135.7, 133.7 (2C), 130.5 (2C), 129.9 (3C), 128.7 (2C), 128.6 (3C), 126.2, 118.8, 116.5, 116.0, 114.8, 65.0, 57.3, 56.6, 53.0. HRMS (ESI+) m/z calcd for $C_{30}H_{20}O_9Na$ $(M+Na^+)$: 547.1011, found: 547.1004.

15. Cyclohexanecarbonyl derivative 12

To a stirred solution of **1** (20.0 mg, 63.2 μmol) in DCM (632 μL, 0.1 M) were added Et_3N (29.1 μL, 208.6 μmol) and cyclobutanecarbonyl chloride (27.9 μL, 208.6 μmol) at room temperature. After being stirred for 12 hr, the reaction mixture was quenched with sat. NH_4Cl aq. and diluted with AcOEt. The resulting mixture was extracted with AcOEt and washed with brine. The organic phase was dried over Na_2SO_4, concentrated under the reduced pressure. The residue was purified by PTLC (hexane : AcOEt = 1 : 2) to give cyclobutanecarbonyl derivative **11** (24.0 mg, 89%) as a yellow solid. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 12.18 (s, 1H), 7.01 (dd, J=9.9, 3.8 Hz, 1H), 6.87 (s, 1H), 6.79 (s, 1H), 6.74 (dd, J=9.9, 1.2 Hz, 1H), 5.13 (s, 2H), 4.11 (dd, J=3.8, 1.2 Hz, 1H), 3.88 (s, 3H), 2.43–2.37 (m, 1H), 1.98–1.21 (m, 10H); and ^{13}C-NMR (125 MHz, $CDCl_3$) δ 180.4, 175.6, 167.2, 160.9, 159.6, 155.4, 145.8, 136.7, 126.3, 113.5, 110.6, 109.8, 105.6, 64.7, 57.3, 56.6, 53.3, 43.2, 29.1 (2C), 25.8, 25.5 (2C). HRMS (ESI+) m/z calcd for $C_{23}H_{23}O_8$ $(M+H^+)$: 427.1393.

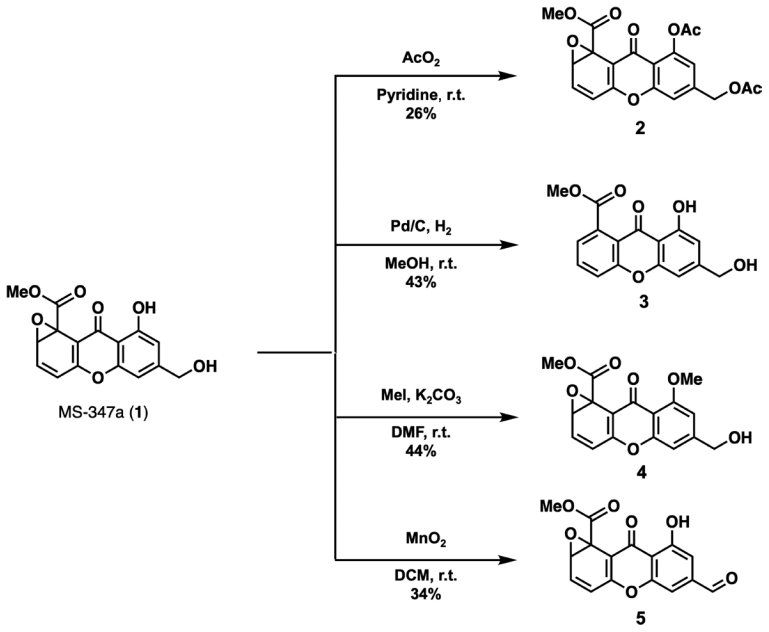
16. Evaluation of antifungal activity

The method was performed according to the protocols in a previous study.[6] The following microorganisms were used for the antifungal activity evaluation on a paper disk method: *Pyricularia oryzae* APU15-60A (quinone outside inhibitors-sensitive [QoIS]), *P. oryzae* APU15-63A (quinone outside inhibitors-resistant [QoIR]), *Botrytis cinerea* MAFF-306820, *Leptosphaeria maculans* MAFF-726728, *Rizoctonia solani* MAFF-237699, and *Colletotrichum gloeosporioide* MAFF-237219 strains.[7–11] The QoIS and QoIR strains were collected from the paddy field of Akita Prefecture in 2015. Compound **1** was prepared as 10 mg/mL DMSO solution. Each paper disk (diameter 6 mm, thin type, Advantec, Tokyo, Japan) impregnated with 10, 3, 1, 0.3, 0.1, 0.03, and 0.01 μg of compounds were put into an agar plate. The *B. cinerea* MAFF-306820, *L. maculans* MAFF-726728, *C. gloeosporioide* MAFF-237219, *R. solani* MAFF-237699, QoIS, and QoIR strains were each grown on PSA solid medium (0.5% potato peptone, 2% sucrose, 0.05% L-glutamic acid Na, and 2% agar). Sterile filter disks impregnated with each compound solution were placed on the agar plate, and the plates were incubated at 25°C for 3 days. After incubation, the diameter (mm) of the inhibition zone was measured.

Results and discussion

Our investigation commenced with the acquisition of **1** by fermentation of the *laeA* gene introduced mutant strain, KTF-0058, which was established by us.[5] This *laeA* gene introduction strategy enabled us to obtain **1** in significantly greater quantities (180 mg/kg). With sufficient quantities of **1** for derivatization in hand, we tackled the synthesis of derivatives as shown in Scheme 1 and Scheme 2. As a first step, acylation of **1** with acetic anhydride and pyridine provided acetyl derivative **2** in 26% yield. In this transformation, undesired side reactions such as aromatization occurred simultaneously, resulting in the low yield of **2**. This result made us realize the instability of the epoxy moiety even under relatively mild conditions, which would be attributed to an energetically less favored dearomatized xanthone structure. Therefore, we tried hydrogenation of the olefin moiety with Pd/C and H_2 to prevent the undesired aromatization reactions. However, unexpected deoxygenative aromatization proceeded to give **3** in 43% yield, suggesting that the epoxy cyclohexadiene ring would be unstable also under the hydrogenation conditions. From this result, we decided to derivatize the 3-hydroxybenzyl alcohol moiety. Selective methylation of the phenol moiety under K_2CO_3 and MeI-used conditions furnished methyl derivative **4** in 44% yield. Subsequently, oxidation of the primary alcohol utilizing MnO_2 gave aldehyde derivative **5** in 34% yield.

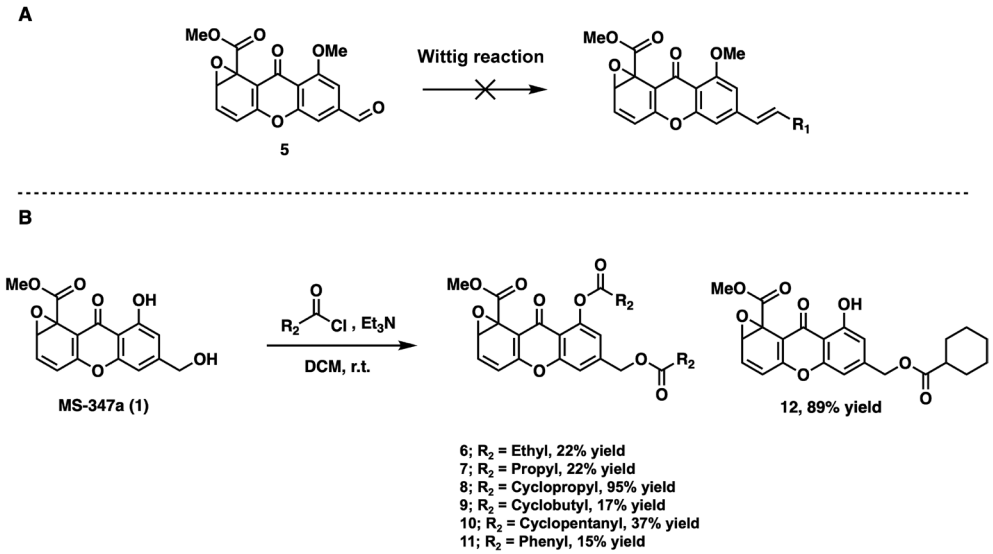
In order to verify the structure–activity relationship of **1**, we started by evaluating the antifungal activity of four derivatives against pathogenic plant fungi (Table 1). Compound **3** and **4** lost the activity, whereas **2** retained the efficacy against the six different fungal strains. It is noteworthy that **5** showed more potent fungicidal activity compared to the parent compound, approxi-



Scheme 1. Synthesis of derivatives-**2**–**5**.

mately 2–30 times higher potency. Considering the antifungal activity of **1** and **3**, the epoxy moiety of **1** appears to be a very important functional group. Considering the labile epoxy moiety and the activity of those derivatives, the oxirane might bind covalently to the molecular target(s), resulting in showing corresponding fungicidal activity. In contrast to **1**, methyl derivative **4** turned out to be not active against all 6 fungal strains due to the extremely low solubility of the compound in water and organic solvents such as DMSO. On the other hand, since bisacetylated derivative **2** showed a respectable level of the activity, the electron deficient chromone ring somewhat increase the activity. This is probably due to the electron density of the chromone moiety affects the electrophilicity of the oxirane. In these regards, further investigations are needed to identify the molecular target(s) and prove our hypothesis.

Next, we sought to synthesize more derivatives based on **2** and **5** to gain more insight into the structure–activity relationship. Considering the instability of the epoxy moiety in **5**, we chose the Wittig reaction using isolated ylid reagents (Scheme 2A), which do not need any additives such as bases. Unfortunately, aromatized products were obtained instead of the desired homologated products, suggesting that the epoxy moiety is more



Scheme 2. Synthesis of derivatives-**6**–**12**.

6; R_2=Ethyl (22% yield), **7**; R_2=Propyl (22% yield), **8**; R_2=Cyclopropyl (95% yield), **9**; R_2=Cyclobutyl (17% yield), **10**; R_2=Cyclopentanyl (34.0% yield), **11**; R_2=Phenyl (32.0% yield), **12**; 89% yield.

Table 1. Antifungal activity of **2**–**5** against phytopathogenic fungi

Strain	Compound	Inhibition zone diameter (mm)					
		*μ*g/disk					
		10	3	1	0.3	0.1	0.03
Pyricularia oryzae APU15-60A (QoIS)	**1**	24.19	16.62	12.46	8.80	—	—
	2	23.13	17.62	12.11	8.73	—	—
	3	10.68	—	—	—	—	—
	4	—	—	—	—	—	—
	5	19.37	16.43	12.81	10.43	8.38	—
	AMPH	N.T.	N.T.	12.46	N.T.	10.40	N.T.
	KXM	N.T.	N.T.	29.14	N.T.	N.T.	N.T.
Colletotrichum gloeosporioides MAFF-237219	**1**	8.64	7.73	—	—	—	—
	2	7.98	—	—	—	—	—
	3	—	—	—	—	—	—
	4	—	—	—	—	—	—
	5	10.52	8.99	7.48	7.06	—	—
	AMPH	N.T.	N.T.	12.28	N.T.	N.T.	N.T.
Leptosphaeria maculans MAFF-726728	**1**	18.70	13.89	9.67	—	—	—
	2	12.05	8.87	—	—	—	—
	3	—	—	—	—	—	—
	4	—	—	—	—	—	—
	5	14.03	12.35	9.57	8.49	—	—
	AMPH	N.T.	N.T.	12.45	N.T.	N.T.	N.T.
Pyricularia oryzae APU15-63A (QoIR)	**1**	20.54	14.97	12.29	8.17	—	—
	2	27.82	20.63	13.27	9.43	—	—
	3	—	—	—	—	—	—
	4	—	—	—	—	—	—
	5	19.50	18.90	13.28	11.49	9.29	—
	AMPH	N.T.	N.T.	14.81	N.T.	N.T.	N.T.
	KXM	N.T.	N.T.	—	N.T.	N.T.	N.T.
Botrytis cinerea MAFF-306820	**1**	15.58	—	—	—	—	—
	2	15.29	2.55	—	—	—	—
	3	—	—	—	—	—	—
	4	—	—	—	—	—	—
	5	14.01	11.52	10.40	9.28	—	—
	AMPH	N.T.	N.T.	10.25	N.T.	N.T.	N.T.
Rizoctonia solani MAFF-237699	**1**	11.06	8.56	—	—	—	—
	2	12.11	—	—	—	—	—
	3	—	—	—	—	—	—
	4	—	—	—	—	—	—
	5	10.13	9.92	7.67	—	—	—
	AMPH	N.T.	N.T.	7.73	N.T.	N.T.	N.T.

AMPH, Amphotericin B; KXM, Kresoxim-methyl. N.T., Not tested; —, no inhibition. *Pyricularia oryzae* APU15-60A, Susceptible to QoI; *Pyricularia oryzae* APU15-63A, Resistance to QoI.

reactive than the aldehyde moiety even under the mild nucleophilic conditions. With the purpose of increasing the reactivity of the aldehyde moiety, we raised reaction temperature, which was also unfruitful. This situation prompted us to conduct acylation using several acid chloride or anhydride reagents for the synthesis of bisacyl derivatives such as **2**. We synthesized six kinds of acyclic and cyclic bisacyl derivatives (Scheme 2B). In the case of cyclohexanecarbonyl chloride-used reaction, the mono acylated product **12** was obtained even with excess amount of the reagent. We evaluated the antifungal activity of **6**–

Table 2. Antifungal activity of **6**–**12** against phytopathogenic fungi

Strain	Compound	Inhibition zone diameter (mm)					
		*μ*g/disk					
		10	3	1	0.3	0.1	0.03
Pyricularia oryzae APU15-60A (QoIS)	**1**	23.12	17.40	12.04	10.29	7.12	—
	6	17.24	13.20	10.51	8.46	—	—
	7	7.95	11.33	11.20	7.79	—	—
	8	26.30	17.50	14.86	14.39	11.28	—
	9	16.45	16.16	11.03	10.84	—	—
	10	—	—	—	—	—	—
	11	11.45	9.07	7.58	7.01	—	—
	12	20.75	14.72	12.31	11.46	—	—
	AMPH	N.T.	N.T.	17.82	N.T.	N.T.	N.T.
	KXM	N.T.	N.T.	20.32	N.T.	N.T.	N.T.
Colletotrichum gloeosporioides MAFF-237219	**1**	9.40	8.57	7.92	—	—	—
	6	7.98	6.90	6.75	—	—	—
	7	6.96	7.18	6.94	6.76	—	—
	8	10.92	8.31	8.18	—	—	—
	9	8.83	8.54	6.94	—	—	—
	10	—	—	—	—	—	—
	11	6.99	6.52	6.09	—	—	—
	12	7.47	7.58	7.30	6.92	—	—
	AMPH	N.T.	N.T.	9.90	N.T.	N.T.	N.T.
Leptosphaeria maculans MAFF-726728	**1**	18.50	13.47	8.40	—	—	—
	6	8.87	7.82	7.30	—	—	—
	7	—	—	—	—	—	—
	8	9.76	8.60	8.10	—	—	—
	9	—	—	—	—	—	—
	10	—	—	—	—	—	—
	11	7.32	—	—	—	—	—
	12	8.44	7.40	6.76	—	—	—
	AMPH	N.T.	N.T.	13.77	N.T.	N.T.	N.T.
Pyricularia oryzae APU15-63A (QoIR)	**1**	14.49	8.39	7.37	6.99	—	—
	6	11.72	10.52	7.56	6.94	—	—
	7	6.92	7.82	7.59	6.46	—	—
	8	14.22	11.32	7.53	—	—	—
	9	7.30	7.04	—	—	—	—
	10	—	—	—	—	—	—
	11	8.05	7.33	6.58	6.12	—	—
	12	8.02	8.02	7.71	6.88	8.40	—
	AMPH	N.T.	N.T.	9.87	N.T.	N.T.	N.T.
	KXM	N.T.	N.T.	—	N.T.	N.T.	N.T.
Botrytis cinerea MAFF-306820	**1**	12.36	10.80	9.63	—	—	—
	6	9.32	8.39	—	—	—	—
	7	—	—	—	—	—	—
	8	10.88	9.58	8.40	—	—	—
	9	—	—	—	—	—	—
	10	—	—	—	—	—	—
	11	7.75	—	—	—	—	—
	12	9.49	9.46	7.69	7.22	—	—
	AMPH	N.T.	N.T.	9.30	N.T.	N.T.	N.T.
Rizoctonia solani MAFF-237699	**1**	18.30	11.36	—	—	—	—
	6	12.11	8.90	7.05	—	—	—
	7	—	—	—	—	—	—
	8	13.77	11.36	—	—	—	—
	9	12.36	9.01	—	—	—	—
	10	—	—	—	—	—	—
	11	8.71	—	—	—	—	—
	12	8.98	7.71	—	—	—	—
	AMPH	N.T.	N.T.	11.47	N.T.	N.T.	N.T.

AMPH, Amphotericin B; KXM, Kresoxim-methyl. N.T., Not tested; —, no inhibition. *Pyricularia oryzae* APU15-60A, Susceptible to QoI; *Pyricularia oryzae* APU15-63A, Resistance to QoI.

12 (Table 2). For QoI-sensitive (QoIS) (*P. oryzae* APU15-60A), QoI-resistant (QoIR) (*P. oryza* APU15-63A), and *Colletotrichum gloeosporioide* MAFF-237219, all compounds except **10** demonstrated antifungal activities comparable to the parent compound, which suggested that several acyl groups are tolerated for the chemical modifications. Intriguingly, **7** exhibited great antifungal activity against QoIS, QoIR, and *Colletotrichum gloeosporioide* but not against the other strains, *Botrytis cinerea* MAFF-306820, *Leptosphaeria maculans* MAFF-726728, and *Rizoctonia solani* MAFF-237699, which implies that the one or two carbons length of the alkyl chain in the acyclic acyl type would be appropriate for broad antifungal activity. In case of the efficacy against *Botrytis cinerea* MAFF-306820, *Leptosphaeria maculans* MAFF-726728 strains, **6**, **8**, and **12** displayed satisfactory biological activities. Compounds **6**, **8**, and **12** demonstrate broad antifungal activity against all six strains, whereases **9** did not show antifungal activity against *Botrytis cinerea* MAFF-306820 and *Leptosphaeria maculans* MAFF-726728, suggesting that the suitable ring size of the acyl group would be three and six. However, cyclohexanecarbonyl derivative **12** is a mono acyl type compound and the number of acyl groups might influence its antifungal activity. Additionally, cyclopentenoyl derivative **10** did not show any antifungal activities against the 6 strains. This anomalous result does not fit in with the tendency of the other results.

In conclusion, we have previously identified **1** as a potent antifungal compound against broad pathogenic plant fungal species after utilizing the *laeA* gene introduction strategy which allowed for sufficient supply of **1** for the structure–activity relationship study. To this end, we synthesized 11 derivatives and evaluated their antifungal activity. Among them, **5** showed more potent activity compared to **1** and acyl derivatives **2**, **6**, **8, 12** maintained comparable activity. From this investigation, it is found that **5** would be one of the most promising derivatives and the derivatization with acyl groups would be a useful approach to create chemical probes for target identification experiments. We are planning to conduct *in vivo* tests using **1** and **5** against pathogenic plant fungi and synthesize chemical probes from **1** *via* acylation for the target identification.

Conflicts interests

The authors declare no competing financial interest.

Author's contributions

Aoi. K., S. H., Atsushi. K., P.W., and Y. A. conceptualized and directed the project. H. K., T. T., A. S., Y. A., T. T., and K. I. constructed and maintained *Aspergillus* sp. S. H., H. K., T. T., and A. S., isolated MS-347a. Aoi. K. and Atsushi. K. synthesized derivatives. S. H., Aoi. K., A. N., M. H., S. K., T. T., T. C., T. U., and S. F. performed the biological experiments. Aoi. K., S. H., Atsushi K., P. W., and Y. A. drafted the manuscript with the assistance from all co-authors. All authors approved the final manuscript. Aoi. K., S. H., and Atsushi K. contributed equally.

Data availability statement

The data underlying this article are available in the article and in its online supplementary material.

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Electronic supplementary materials

The online version of this article contains supplementary material, which is available at https://www.jstage.jst.go.jp/browse/jpestics/.

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